Journal of Chromatography A, 659 (1994) 87–93
Elsevier Science B.V., Amsterdam

CHROM. 25 558

High-performance liquid chromatographic separations of naphthoquinones and their derivatives

Effect of hydrogen bonding on retention

Wenche Stensen and Einar Jensen*

Department of Plant Physiology and Microbiology, IBG, University of Tromsø, N-9037 Tromsø (Norway)

(First received May 17th, 1993; revised manuscript received September 14th, 1993)

ABSTRACT

A method for the separation and determination of fifteen naphthoquinone derivatives was developed, based on reversed-phase high-performance liquid chromatography and ultraviolet–visible detection. The effect on the selectivity of different mobile phase compositions (*e.g.*, methanol–water and acetonitrile–water binary mixtures and methanol–acetonitrile–water ternary mixture) was investigated. The retention order of the compounds with methanol–water as eluent is interpreted on the basis of intramolecular hydrogen bonding in the solute *versus* intermolecular hydrogen bonding between the solute and the solvent. The hydrogen bonding pattern was studied using quantum chemical calculations.

INTRODUCTION

It is well documented that many 1,4-naphthoquinone derivatives show antimicrobial activity, especially if a hydroxy group is present at the C-5 position [1]. A chlorine substituent in the quinone ring also increases the activity of 1,4-naphthoquinones [2,3], and dichlone (2,3-dichloro-1,4-naphthoquinone) is a well known agricultural fungicide [4].

We have recently tested the effects of several naphthoquinones on some common fish pathogenic bacteria, *Aeromonas salmonicida*, *Vibrio salmonicida* and *V. anguillarum*, and also *Escherichia coli*. The results indicated that very low concentrations (<1 μg/ml) of some of the tested compounds inhibit the growth of the pathogenic bacteria, but have no effect on *E. coli* in the tested concentration range [5]. In order to evaluate the potential use of these naphthoquinones as antibacterial compounds in problems related to fish farming, chromatographic methods for the determination of different naphthoquinones had to be established.

There are few reports describing chromatographic separations and determinations of non-isoprenoid naphthoquinones. In a study of the metabolism of plumbagin in rats, a method based on thin-layer chromatography has been used [6]. Marston and Hostettmann [7] demonstrated the separation of six different naturally occurring naphthoquinones using a μBondapak CN column, and Rittich and Krska [8] used Micro Pak Si-10 and CN-10 columns in an attempt to separate a mixture of quinones.

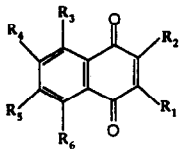
We wanted to find analytical methods for a subsequent study of some of the naphthoquinones and their metabolites in a biological matrix. However, none of the above-mentioned methods seemed to be appropriate in this respect. We therefore found it necessary to

* Corresponding author.

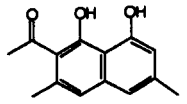
0021-9673/94/$07.00
SSDI 0021-9673(93)E0999-B

develop methods for the separation and determination of a wide range of natural and synthetic naphthoquinone derivatives (Fig. 1). In general, a metabolite is more polar than its parent substance [9], so on a reversed-phase column metabolites are expected to elute before the substrate as retention time decreases with increasing polarity. With this in mind, we decided to use a reversed-phase C_{18} column.

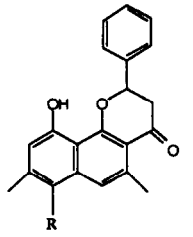
During the work we observed that the retention order of some of the compounds was the opposite of what was expected from a superficial understanding of retention mechanisms in reversed-phase systems. In order to explain the observed results, we used semi-empirical molecular orbital calculations to derive the hydrogen bonding patterns for some typical naphthoquinones.



Compound	R1	R2	R3	R4	R5	R6
1	H	H	H	H	H	H
2	H	H	OH	H	H	H
3	H	H	OH	H	H	OH
4	CH_3	H	OH	H	H	H
5	CH_3	H	OH	H	CH_3	H
6	CH_3	H	OCH_3	H	CH_3	H
7	CH_3	CH_3	OH	H	CH_3	H
8	CH_3	CH_2CH_3	OH	H	CH_3	H
9	CH_3	H	OH	CH_2CH_3	CH_3	H
10	CH_3	H	OH	CH_3CO	CH_3	H
11	CH_3	H	OH	H	CH_3	CH_3CO
12	CH_3	H	OCH_3	CH_3CO	CH_3	H



13



Compound	R
14	H
15	Cl

Fig. 1. Structures of naphthoquinone derivatives.

EXPERIMENTAL

Methanol and acetonitrile, obtained from Lab-Scan and Fluka, respectively, were of HPLC grade. Water was distilled and filtered through a 0.45-μm Millipore filter.

5-Hydroxy-1,4-naphthoquinone (juglone) (**2**) and 5-hydroxy-2-methyl-1,4-naphthoquinone (plumbagin) (**4**) were purchased from Aldrich. 1,4-Naphthoquinone (**1**), 5,8-dihydroxy-1,4-naphthoquinone (naphtazarin) (**3**), 5-hydroxy-2,7-dimethyl-1,4-naphthoquinone (**5**), 5-methoxy-2,7-dimethyl-1,4-naphthoquinone (**6**), 5-hydroxy-2,3,7-trimethyl-1,4-naphthoquinone (**7**), 3-ethyl-5-hydroxy-2, 7-dimethyl-1,4-naphthoquinone (**8**), 6-ethyl-5-hydroxy-2,7-dimethyl-1,4-naphthoquinone (**9**), 6-acetyl-5-hydroxy-2,7-dimethyl-1,4-naphthoquinone (**10**), 8-acetyl-5-hydroxy-2,7-dimethyl-1,4-naphthoquinone (**11**), 6-acetyl-5-methoxy-2, 7-dimethyl-1, 4-naphthoquinone (**12**), 2-acetyl-3,6-dimethyl-1,8-naphthalenediol (**13**), 10-hydroxy-5,8-dimethyl-2-phenyl- 2,3- dihydronaphtho[1,2-*b*]pyran- 4 -one (**14**), and 7-chloro-10-hydroxy-5,8-dimethyl-2-phenyl -2,3- dihydronaphtho[1,2-*b*]pyran- 4 -one (**15**) were generously donated by Dr. J.C. Overeem, TNO Research Institute, Zeist, Netherlands.

Solutions of naphthoquinones in methanol for HPLC analysis were in the concentration range 0.05–0.10 μg/μl, and 10–15 μl were injected on to the column. The mobile phase was a combination of the following solutions: (a) methanol with 30 m*M* acetic acid, (b) water with 30 m*M* acetic acid and (c) acetonitrile with 30 m*M* acetic acid.

Separations were performed on a Waters HPLC system consisting of a Model 600 E multisolvent delivery system, a column heater, a Model 712 Wisp autoinjector, a Model 486 absorbance detector operated at 415 nm and a Model 746 integrator. A Waters Nova-Pak 4-μm C_{18} reversed-phase column (150 × 3.9 mm, I.D.) operated at 30°C was used. The flow-rate was 1 ml/min. UV–Vis spectra were recorded on a Shimadzu UV 160 spectrophotometer.

Semi-empirical molecular orbital calculations were performed on a Silicon Graphics Personal Iris 4D/30 EG computer using the programs Quanta 3.3/CHARMm 22 [10] and MOPAC v

5.00 [11]. Starting conformations for the quantum mechanical calculations were obtained from CHARMm-optimized structures. The semi-empirical calculations were performed using the AM1 Hamiltonian with full geometry optimization.

RESULTS AND DISCUSSION

All the naphthoquinones and their derivatives were yellow to reddish powders or crystalline needles. They absorbed visible light relatively strongly and by studying their UV–Vis spectra (Table I), 415 nm was found to be a suitable wavelength for chromatographic detection. This wavelength was convenient because our experience indicated that with a biological sample, less interference from other substances in the matrix can be expected the longer is the wavelength used.

TABLE I

UV–VIS SPECTROSCOPIC DATA

The compounds were dissolved in 96% ethanol.

Compound	λ (nm)	Log ε	Compound	λ (nm)	Log ε
1	274	3.46	**9**	277	3.92
	333	3.50		415[a]	3.65
	415[a]	2.26		434	3.68
2	271	3.55	**10**	278	4.02
	415[a]	3.56		415[a]	3.88
	423	3.57			
3	280	3.92	**11**	277	4.01
	415[a]	3.37		415[a]	3.66
	488	3.88		425	3.68
4	276	3.87	**12**	273	3.91
	415[a]	3.77		357	3.60
				415[a]	2.71
5	276	3.90	**13**	275	3.89
	415[a]	3.62		347	3.73
	423	3.63		415[a]	3.32
6	274	3.99	**14**	276	3.89
	400	3.77		389	3.93
	415[a]	3.71		415[a]	3.46
7	282	4.06	**15**	275	4.11
	415[a]	3.70		393	3.83
	422	3.71		415[a]	3.49
8	281	4.04			
	415[a]	3.61			
	422	3.62			

[a] Wavelength used for chromatographic detection.

Isocratic elution

First, a study of the chromatographic behaviour of the naphthoquinones and their derivatives was carried out with different isocratic methanol–water compositions as mobile phase. Table II gives the retention times of the compounds listed in order of elution. With the system used, *i.e.*, a reversed-phase column and an aqueous mobile phase, the most polar solutes are least retained. The observed order of elution was as expected for some of the compounds; naphthoquinones **2**, **4**, **5** and **7** with no, one, two and three methyl groups, respectively, eluted in order of increasing lipophilicity. However, the elution order was unexpected in some series. For

TABLE II

CAPACITY FACTORS (k') IN DIFFERENT ISOCRATIC ELUTIONS WITH METHANOL–WATER AS MOBILE PHASE

Compound	k'						
	Methanol concentration (%)						
	40	50	55	60	65	70	80
1	4.0	1.9	1.4	1.0	0.7	0.5	–
2	5.9	2.8	2.0	1.1	1.1	0.8	–
11	10.4	4.1	2.7	1.8	1.2	0.9	–
3	–	4.0	2.9	2.3	1.6	1.2	–
6	12.5	4.6	3.0	2.0	1.3	0.9	–
12	13.2	4.8	3.0	2.0	1.3	0.9	–
4	15.3	6.6	4.5	3.1	2.1	1.5	–
10	–	9.2	5.7	3.6	2.4	1.6	–
5	–	–	9.0	5.9	3.9	2.6	–
13	–	–	16.3	9.1	5.4	3.2	1.6
7	–	–	21.5	13.0	8.2	5.2	2.3
9	–	–	–	–	12.7	7.7	3.0
8	–	–	–	–	13.8	8.3	3.2
14	–	–	–	–	19.0	13.5	4.0
15	–	–	–	–	–	28.2	8.2

instance, compounds **1**, **2** and **3** with no, one and two hydroxyl groups, respectively, would be expected on the basis of the number of polar functional groups to elute in the opposite order. In addition, the regioisomers **10** and **11** separated unexpectedly well, in contrast to **8** and **9**, which eluted with similar retentions.

The precise mechanism of retention is difficult to describe owing to the complexity of the possible mechanisms, and various theories have been put forward pointing out which interactions are important in reversed-phase chromatography [12–14]. The general opinion appears to be that interactions between solute and mobile phase are of great importance. For polar molecules in a polar solvent the most powerful interaction is hydrogen bonding. The mobile phase in our system consisted of polar protic solvents that are able to form strong hydrogen bonds. The solutes are all hydrogen bond acceptors (carbonyl and hydroxyl groups) and in addition some are hydrogen bond donors (hydroxyl groups), hence all the compounds are able to form hydrogen bonds with the solvents. However, naphthoquinones with hydroxyl groups in *peri* positions to the carbonyl functions are able to form strong intramolecular hydrogen bonds, creating a six-membered ring. It is well known that most intramolecular hydrogen bonding occurs when six-membered rings can be formed [15]. There will then be a competition between intramolecular (solute–solute) and intermolecular (solute–solvent) hydrogen bonding, where in general the intramolecular interaction will win. A classical example of this relationship is seen in nitrophenols: *o*-nitrophenol, which forms an intramolecular hydrogen bond, is eight times less soluble in water than the *para* isomer [16]. The effect of intramolecular solute hydrogen bonding on retention on silica and alumina stationary phases has been discussed previously [17].

Theoretical energy calculations of the naphthoquinones with a hydroxyl group in a *peri* position show an internal hydrogen bond that is approximately three times stronger than the binding energy between two water molecules [18]. Experimental evidence for the presence of a strong hydrogen bond in similar systems has also been reported using NMR and IR spectroscopy [19,20], adsorption chromatography [21] and liquid–liquid chromatography [22].

On the basis of the theory that the intermolecular solute–solvent hydrogen bonding interaction is the most important retention mechanism, the order of elution is easier to explain. For the naphthoquinone series **1**, **2** and **3**, **1** has two free carbonyl groups that can act as hydrogen bond acceptors for water and methanol. The interaction is consequently large and the compound distributes easily in the mobile phase and will thus be very little retained. Naphthoquinone **3**, on the other hand, has two strong intramolecular hydrogen bonds, rendering the two quinonoid carbonyls much less efficient hydrogen bond acceptors, and hence it dissolves less efficiently in the mobile phase and is more retained than **1**. Naphthoquinone **2**, with one intramolecular hydrogen bond and one free carbonyl group, shows intermediate retention.

The same argument can be used to explain the relative retention between **10** and **12** and between **5** and **6**. Naphthoquinones **10** and **12** have the same structure except that the hydroxyl group in **10** is replaced by a methoxy group in **12**. The expected effect of this should be that **12** is less polar than **10** and would therefore be more retained, which is the opposite of what is observed. The reason for this is again found in intramolecular hydrogen bonding. Naphthoquinone **10** forms an intramolecular hydrogen bond which is precluded by the methoxy group in **12**. The result is that **12** has two free hydrogen bond acceptor carbonyls whereas **10** has only one. Consequently, **12** dissolves more easily in the mobile phase and is less retained than **10**. The effect of breaking the intramolecular hydrogen bond by a methoxy group is likewise seen in the retentions of **5** and **6**.

Isomeric compounds often show similar retentions in reversed-phase chromatography [23], as we observed for **8** and **9**. In this context, the retention difference between **10** and **11** is striking; **11** is much less retained than **10**. Although a hydrogen bond between the hydroxyl group and the acetyl function can be envisaged for **10**, calculations show this to be of minor importance.

TABLE III

CAPACITY FACTORS (k') AND RESOLUTION (R_s) FOR NON-BASELINE-SEPARATED PEAKS WITH GRADIENT ELUTION

Gradient A, methanol–water: linear gradient from 40% to 80% methanol in 40 min followed by isocratic elution with 80% methanol for 10 min. Gradient B, acetonitrile–water: linear gradient from 25% to 75% acetonitrile in 55 min. Gradient C, methanol–acetonitrile–water: linear gradient from 37% to 80% methanol–acetonitrile (93:7) in 43 min followed by isocratic elution with 80% methanol–acetonitrile (93:7) for 10 min.

Compound	k' (R_s)		
	Gradient A	Gradient B	Gradient C
1	4.1	5.8	4.5
2	5.8	7.3	6.3
11	9.2	11.8	10.1
3	7.8	8.8 (1.3)	8.1
6	10.2 (0.5)	9.7 (1.3)	11.1 (1.2)
12	10.6 (0.5)	13.1 (0.4)	11.6 (1.2)
4	11.9	13.5 (0.4)	12.5
10	15.1	17.4	16.0
5	18.2	19.3	18.7
13	23.3	24.6	24.0
7	25.4	25.9	25.7
9	29.5 (1.0)	30.9 (0.6)	30.0 (0.7)
8	30.0 (1.0)	31.2 (0.6)	30.4 (0.7)
14	34.5	37.2	34.9
15	39.9	43.8	39.9

Consequently, the intramolecular hydrogen bonding pattern is expected to be similar for both compounds. One quinonoid carbonyl is occupied in intramolecular hydrogen bonding, leaving the other quinonoid carbonyl and the acetyl free for intermolecular hydrogen bonding in both compounds. The difference in elution order can be explained by their different accessibilities for intermolecular hydrogen bonding. Geometry optimisation of **11** placed the carbonyl in the acetyl group perpendicular to the ring plane, whereas for **10** the carbonyl formed an angle of approximately 40° to ring plane pointing towards the methyl group. The acetyl group is therefore less sterically hindered for hydrogen bonding towards the solvent in **11** than in **10**.

Gradient elution

None of the isocratic methanol–water elutions separated all fifteen compounds (Table II). The pairs of compounds **6–12** and **11–3** eluted nearly simultaneously with methanol–water (1:1). On lowering the methanol content in the mobile phase, **6** and **12** were partly resolved but **3** showed very poor chromatographic properties. Based on Table II, a methanol–water gradient (gradient A in Table III) was defined that separated all the components except **6** and **12** and the regioisomers **8** and **9** (Fig. 2).

It has been demonstrated that replacing meth-

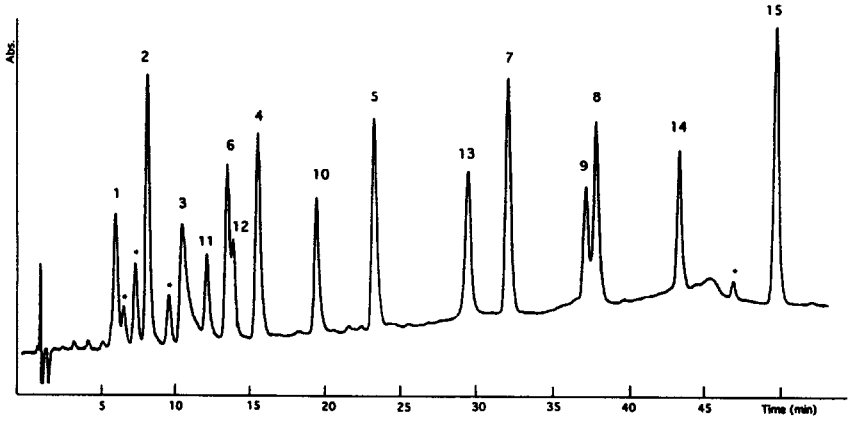


Fig. 2. HPLC separation of naphthoquinones with a linear methanol–water gradient (gradient A in Table III). The relative concentrations are not identical in the chromatograms. Asterisks indicate impurities.

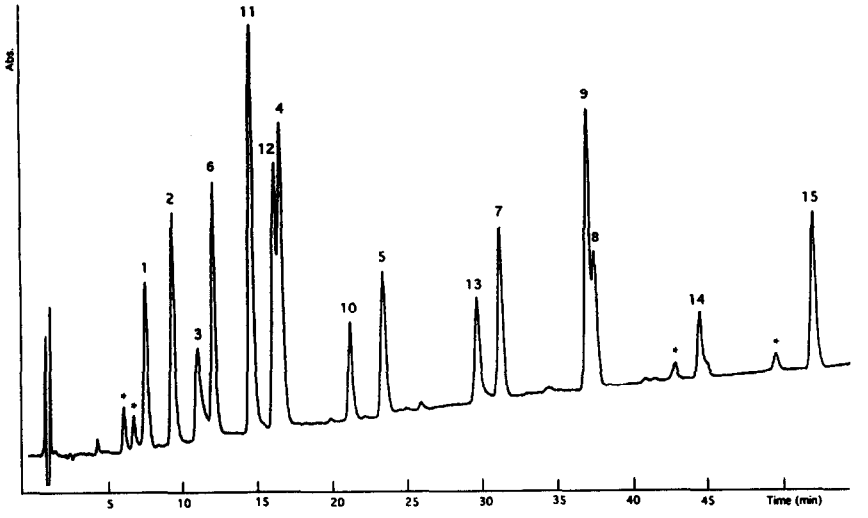


Fig. 3. HPLC separation of naphthoquinones with a linear acetonitrile–water gradient (gradient B in Table III). The relative concentrations are not identical in the chromatograms. Asterisks indicate impurities.

anol with acetronitrile in the mobile phase gives a slightly different selectivity towards compounds that are difficult to separate in methanol–water systems [24]. Employing this principle, a linear acetonitrile–water gradient (gradient B in Table III) was applied and baseline separated compounds **6** and **12**. However, the separation between the regioisomers **9** and **8** and between **12** and **4** decreased (Fig. 3).

Therefore, a ternary gradient composed of methanol, acetonitrile and water was constructed (gradient C in Table III). This system gave an acceptable solution to the separation problem, although **6** and **12** were not baseline separated

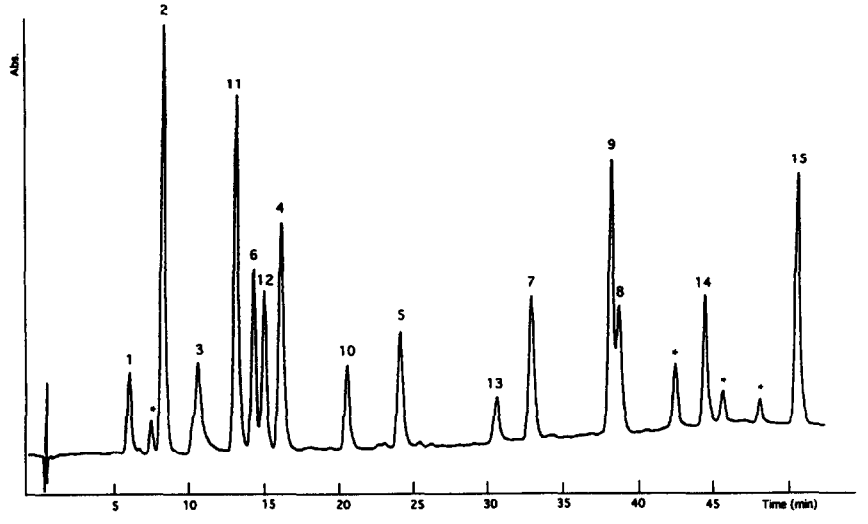


Fig. 4. HPLC separation of naphthoquinones with a ternary gradient composed of methanol–acetonitrile–water (gradient C in Table III). The relative concentrations are not identical in the chromatograms. Asterisks indicate impurities.

(resolution 1.2) (Fig. 4). In addition, the regioisomers **9** and **8** were not adequately resolved, but this pair could be separated in an additional isocratic analysis with methanol–water (65:35) (Table II).

CONCLUSIONS

Chromatographic methods employing a ternary gradient were developed that separated thirteen of fifteen naphthoquinones and naphthoquinone derivatives; the two remaining compounds had to be separated in an additional step. Hydrogen bonding between the solute and the solvent can explain the observed retention with methanol–water eluents. Intramolecular hydrogen bonding in the solute led to less solubility in the eluent resulting in an increase in the retention. Consequently, compounds with polar substituents will not necessarily be less retained on a reversed-phase column. This result indicates that the assumption that the metabolites of the naphthoquinones will have decreased retention compared with the parent substance will not always be true.

ACKNOWLEDGEMENTS

Financial support from the Norwegian Research Council (NFR) is gratefully acknowledged. Thanks are also due to Dr. Jan Raa for introducing us to the field of marine antibiotics, to Dr. Jan C. Overeem for providing the different naphthoquinones and to Dr. John S. Svendsen for performing the calculations.

REFERENCES

1 B.A. Kulkarni, V.D. Kelkar and P.L. Kulkarni, *Indian J. Pharm. Sci.*, 45 (1983) 21.
2 A.V.B. Sankaram, T.N. Srinivasan and A.S. Indulkar, *Pestic. Sci.*, 6 (1975) 165.
3 V. Ambrogi, D. Artini, I. De Carneri, S. Castellino, E. Dradi, W. Logemann, G. Meinardi, M. Di Somma, G. Tosolini and E. Vecchi, *Br. J. Pharmacol.*, 40 (1970) 871.
4 S. Budavari (Editor), *The Merck Index*, Merck, Rahway, NJ, 11th ed., 1989, p. 3032.
5 J. Raa, personal communication.
6 B. Chandrasekaran and B. Nagarajan, *J. Biosci.*, 3 (1981) 395.
7 A. Marston and K. Hostettmann, *J. Chromatogr.*, 295 (1984) 526.
8 B. Rittich and M. Krska, *J. Chromatogr.*, 130 (1977) 189.
9 H.P. Rang and M.M. Dale, *Pharmacology*, Churchill Livingstone, New York, 2nd ed., 1991, p. 91.
10 B.R. Brooks, R.E. Bruccoleri, B.D. Olafson, D.J. States, S. Swaminathan and M. Karplus, *J. Comput. Chem.*, 4 (1983) 187.
11 J.J.P. Stewart and F.J. Seiler, *MOPAC: A General Molecular Orbital Package*, QCPE 455, Department of Chemistry, Indiana University, Bloomington, IN.
12 S.R. Bakalyar, R. McIlwrick and E. Roggendorf, *J. Chromatogr.*, 142 (1977) 353.
13 P.E. Antle, A.P. Goldberg and L.R. Snyder, *J. Chromatogr.*, 321 (1985) 1.
14 J.G. Dorsey and K.A. Dill, *Chem. Rev.*, 89 (1989) 331.
15 J. March, *Advanced Organic Chemistry*, Wiley, New York, 4th ed., 1992, p. 76.
16 R.T. Morrison and R.N. Boyd, *Organic Chemistry*, Allyn and Bacon, Newton, MA, 5th ed., 1987, p. 998.
17 L.R. Snyder, *Principles of Adsorption Chromatography*, Marcel Dekker, New York, 1968, p. 315.
18 L.A. Curtiss and M. Blander, *Chem. Rev.*, 88 (1988) 827.
19 W.-I. Shiau, E.N. Duesler, I.C. Paul, D.Y. Curtin, W.G. Blann and C.A. Fyfe, *J. Am. Chem. Soc.*, 102 (1980) 4546; and references cited herein.
20 G. Wurm and B. Goessler, *Arch. Pharm. (Weinheim, Ger.)*, 322 (1989) 489.
21 S.B. Padhye and B.A. Kulkarni, *Chromatographia*, 8 (1975) 352.
22 J. Franc and J. Sechovec, *J. Chromatogr.*, 212 (1981) 139.
23 L.R. Snyder and J.J. Kirkland, *Introduction to Modern Liquid Chromatography*, Wiley, New York, 2nd ed., 1979, p. 356.
24 E. Jensen, *J. Chromatogr.*, 246 (1982) 126.